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Cryptocaryoniasis of cultured flounder, *Paralichthys olivaceus* in low temperatures

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In the winter of 1995, mass mortality occurred in cultured flounder, *Paralichthys olivaceus* in Gurongpo, Kyoungbuk, Korea. From the observations of moribund and dead fish, parasitic ciliates, which were shown as white spots to the naked eye, were considered to be involved in the mass mortality. From heavily infected flounders, histopathological, morphological and biological characterization of these ciliates were carried out.

In the histological observation, many ciliates were found under the epithelia of gill filaments and skin, and caused hyperplasia of epithelial and mucus cells at the infected areas. The ciliates found on the body surface, fins and gills were very similar to *Cryptocaryon irritans*. However the ciliates showed two different patterns of reproduction, i.e., typical form(palintomy)and atypical form(budding plus multiple fission) at 16℃ of water temperature. The occurrence ratio between typical and atypical form was about 3:2. Tomitogenesis takes 8-14 days in the typical and 13-15 days in the atypical form.

In the viability test at different temperatures and salinities, the typical form died below 30‰ at 12℃, below 20‰ at 16℃, below 15‰ at 20℃, and below 25‰ at 24℃, respectively. On the other hand, the atypical form died below 20‰ at 12℃, below 15‰ at 16-20℃, and below 25‰ at 24℃, respectively. The results suggested that the atypical has better viability at low salinity than that of the typical at low temperatures.

In the excystment time and success rates of excystment according to temperatures, the typical form showed 8 days, 30% at 12℃; 6.5 days, 50%, at 16℃; 5.5 days, 75% at 20℃; and 7 days, 10% at 24℃, respectively. On the other hand, the atypical form showed 15.5 days at 12℃; 14 days, 76.6% at 16℃;12 days, 72.2% at 20℃; 10 days 31.6% at 24℃, respectively. The results suggested that the atypical form had longer excystment time than that of the typical form at any temperature and showed better stability at low temperatures.

Key words : Flounder, *Paralichthys olvaceus*, *Cryptocaryon irritans*, Typical form, Atypical form

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White spot disease of marine fish, caused by the parasitic ciliate *Cryptocaryon irritans* Brown, 1951, was chiefly associated with captive fish in marine aquria(Sikama, 1937; Brown, 1951,; Nigrelli and Ruggieri, 1966; Wilkie and Gordin, 1969). In the last 10 years, however, the disease has emerged as a major problem in the development of mariculture, accounting for high mortalities in cultured food fishes (Huff and Burns, 1981; Kaige and Miyazaki, 1985; Colorni, 1985; Dimant *et al.*, 1991). In Korea, flounder *Paralichthys olivaceus* cultured at several fish farms were often infected by this ciliate(Chun, personal communication).

C. irritans is an obligate parasite of warm-water marine fish where minimum sea temperatures exceed 19℃(Wilkie and Gordin, 1969). The life-cycle of *C. irritans* is direct, growth and development takes place within the fish epidermis, and follows a free-living phase in which the parasite forms a cyst, then undergoes palintomic division and proudces theronts(Brown, 1963; Colorni, 1985; Lom and Dykova, 1992; Burgess and Matthews, 1944). The parasite has generally been regarded in the past as a closely related marine counterpart to *Ichthyophthirius multifiliis*, due mainly to many similarities in their superficial morphologies and life cycles. Because of these similarities, both species have been placed together in the family *Ichthyopthiriidae* by some authors(Nigrelli and Ruggeri, 1966; Corliss, 1975). However recent ribosormal RNA studies have revealed difference between the two species and indicate that they are not closely related as first thought (Diggles and Adlard, 1995). There are some evidence for difference of *C. irritans* strains infecting between Red Sea and Mediterranean fish(Dimant *et al.*, 1991).

In the winter of 1995, white spot disease occurred in cultured flounder, *Paralichthys olivaceus* in Gurongpo, Kyoungbuk, Korea. In order to obtain the information about the outbreaks of white spot disease at low temperatures, histopathological observations using heavily infected flounders and morphological examination of parasitic ciliates were undertaken. Reproductive characteristics and viability of the ciliates were also investigated at different temperatures and salinites.

Materials and Methods

Infected fish

From December 1995 to February 1996, flounder, *Paralichthys olivaceus*, weighing 150-300g which showed white spots to the naked eye were obtained from an embankment flounder farm at Gurongpo. Heavily infected fish were supplied for the histological examination. Fish infected to a low degree were maintained at 14±2℃ and 32±2‰ to investigate morphological and biological characteristics of the parasitic cilliates, and provide for an infection source.

Histopathological examination

Tissues of heavily infected fish were fixed

in 10% netural formalin. Following serial dehydrations with ethanol, tissues were embedded in paraffin wax. Thin sections(4-5㎛ in thickness) were made with a microtome (Rotary type) and stained with hematoxylin and eosin, or Giemsa for light microscopic observation. In observations, parasite size was measured with a micrometer eyepiece.

Morphological features of parasitic ciliates

Each of the infected site was placed in a wet saline mount between a 22-mm² coverslip and a stand glass microscope slide and observed with a light or phase-contrast microscope(40×-100× magnification). Groups of 2 or 3 moribund fish were kept in 30 *l* seawater aquaria(16℃, 32±2‰). Upon dropping off the host, free-swiming ciliates were collected by pipetting and fixed by adding 5% formalin, and observed with a light microscope. To investigate reproduction patterns, the ciliates were transferred to serological plates containing filtered seawater and incubated at 16℃. Every 2 to 3 days they were rinsed with fresh filtered seawater and observed daily with a phase-contrast microscope.

Biological characteristics of parasitic ciliates

Temperature conditions ranging from 12 to 24℃ at 4℃ intervals and saline solutions from 0 to 35 ‰ at 5‰ intervals were used to establish viability and reproductive characteristics of the parasitic ciliate. Salinities below 30‰ were and obtained by diluting seawater with unchlorinated tap water and salinities were confirmed with a Japan Optical(ATAGO) hand refractometer. Free-swiming ciliates released from infected fish were transferred singly to each wells of serological plate and observed daily with a phase-contrast microscope. For the viability study, 4 groups of 160 ciliates that were exposed to each saline solution by 20 individuals were incubated at different temperatures for 14 days. For study of tomite development, 4 groups of 90 ciliates that were exposed to constant salinity(35‰) were examined. Every 2 to 3 days they were rinsed with saline solution or fresh filtered seawater for each experiment. For experimental infections under laboratory conditions, ten healthy, juvenile flounders were exposed in 30 *l* seawater aquarium(16℃, 32±2‰)to 2 or 3 moribund fish.

Results

Infected fish

Multiple white spots with a pin point size were observed in the body surface, fins and gills(Fig. 1). In clinical examination, excessive production of mucus and fading in the gill was seen in affected fish. Sometimes ulcer in the body was observed in moribund fish, but there were no signs of damage in the internal organs.

Histopathological examination

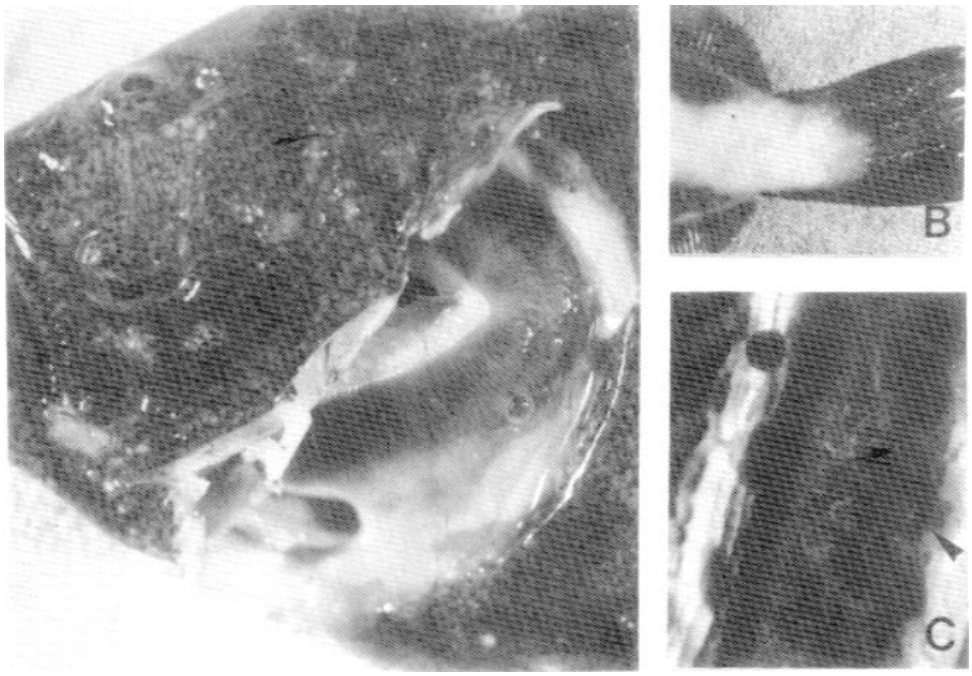

Fig. 1. Many white spots are seen in the gill(A), tails(B) and dorsal fin(C) of diseased flonder. Parasitic ciliates(arrow) are observed with a stereomicroscope(×6.7).

In the histological observations of heavily infected fish, many ciliates were found under the epithelia of gill filament and skin, and caused hyperplasia of epithelial and mucus cells at the parasitic areas(Fig. 2). In the gills, a large ciliate was observed in the blood vessel of a primary gill filament(Fig. 2A). However a small ciliate was found in the interlamellar space in early stage of infcetion and evoked slight epithelial proliferation(Fig. 2B). In the skin, variable size of ciliates were seen in the subepithelia(Fig. 2C), and caused marked hyperplasia of mucus cells and vacuolated degeneration of the basement membrane(Fig. 2D).

Morphological and reproductive features of ciliates

These ciliates showed that the life-cycle is direct including parasitic phase(growth and development within the host) and nonparasitic phase(free swiming and reproduction without the host). The ciliates found on the body surface, fins and gills were whitish in color, 200-700㎛ in size, and spherical or amoeboid in the shape, which had a simple buccal apparatus and developmental contractile vacuoles(Fig. 3). In this respect, these ciliates were very similar to *C. irritans*. From the scrutinized observation of morphometrics and investigation of reprductive patterns, however, the ciliates were differerentiated into two forms, i.e., a typical form of the ciliate which shows the same characteristic as *C. irritans*, and an

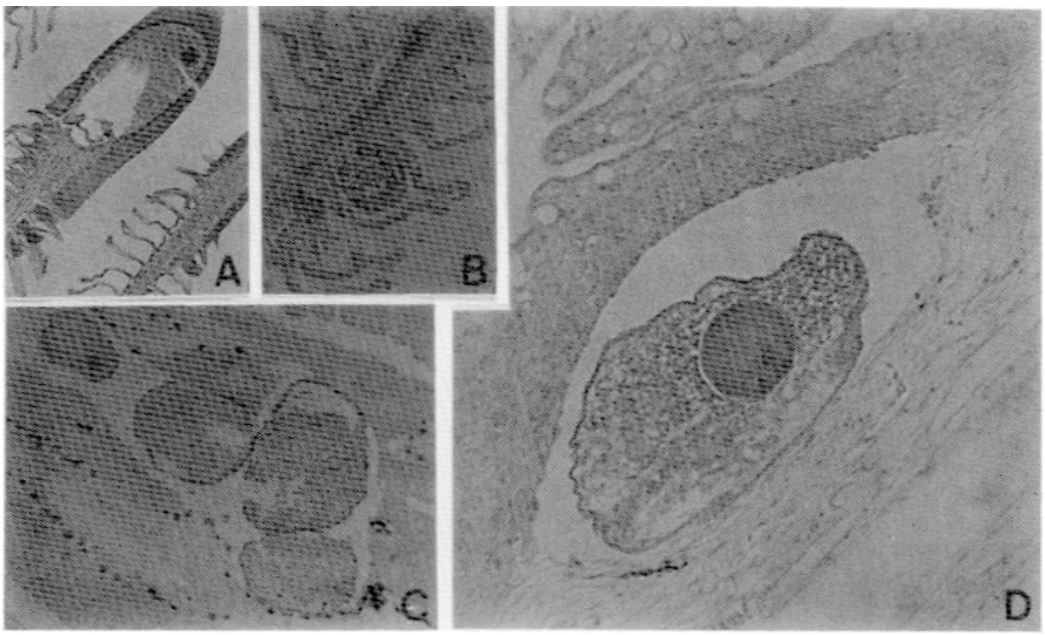

Fig. 2. Cross section of gills and skin in the infected flounder.
A. Parasitic ciliates are found in the blood vesseles of a primary gill filament. Giemsa stain, ×40.
B. A small ciliate infesting interlamellar space. Giemsa stain, ×100.
C. Parasitic ciliates in the skin subepithelium. H & E stain, ×100.
D. Parasitic ciliates in the skin. H & E. stain, ×100.

atypical form of the ciliate which shows different characteristics from *C. irritans*(Table 1). In the size of these ciliates, the atypical form is somewhat larger than typical form. Typical form has four linked moniliform macronuclei, while atypical form has a inconspicuous macronucleus. These morphological differences, however, were not so conspicuous that it was diffcult to distinguish them as a rule. In the reprouduction patterns of the ciliate, typical form takes only a palintomic division(Fig. 4), while atypical form takes a budding and a mulitple fission(Fig. 5).

The occurrence ratio between typical and atypical form was about 3 : 2. Tomitogenesis takes 8-14 days in the typical and 13-15 days in the atypical form(Table 1).

Biological characteristics of ciliates

1. Effects of salinity on parasitic ciliate survival at various temperatures

The results are summarized in Table 2. Both the typical and atypical forms survived at 35‰, and died below 15‰ at all temperatures tested. The typical form died below 30‰ at 12 ℃, below 20‰ at 16℃, below 15‰ at 20℃, below 15‰ at 16-20℃, and below 25‰ at 24

Table 1. Comparison of parasitic ciliates causing cryptocaryoniasis from the present study and the literature

Items	Authority		
	Brown(1951), Nigrelli and Ruggieri (1966), Colorni (1985) and Burgess (1992)	Present	
Organisms	C. irritans	Typical	Atypical[2]
Hosts	Various	Olive flounder	
Temperatures (℃)	20~25	12~16	
Trophont diameter(㎛)	180~450	200~400	350~700
Tomont diameter(㎛)	200~450	200~400	300~700
Theront long(㎛)	40~70	40~60	60~100
Reproduction pattern	Palintomy	Palintomy	Palintomy and budding
Tomitogenesis	3~28 days	8~14 days	13~15 days
Occurrence(%)[3]	-	57.4	42.6

[1] A ciliate showing only palintomic division.
[2] A ciliate showing budding and multiple fission
[3] 100 individuals of ciliates were examined in the present study.

℃, respectively. The results suggested that the atypical has higher viability at low salinity than that of typical in low temperatures.

2. Effects of temperature on tomite development at constant salinity(35‰)

The results are summarized in Table 3. Both the typical and atypical forms showed tomite development at all temperatures tested. In the optimum temperature for tomite development, the typical form was 20℃ and the atypical form was 16℃. In the excystment time and sucess rates of excystment according to temperatures, the typical form showed 8 days, 30% at 12℃; 6.5 days, 50% at 16℃; 5.5 days, 75% at 20℃;and 7 days, 10% at 24℃, respectively. On the other hand, the atypical form showed 15.5 days at 12℃; 14 days, 76.6% at 16℃; 12 days, 72.2% at 20℃; 10 days, 31.6% at 24℃, respectively. The results suggested that the atypical form had longer excystment time than that of typical form at all temperatures and showed better stability in low temperatures.

3. Fish susceptibilty

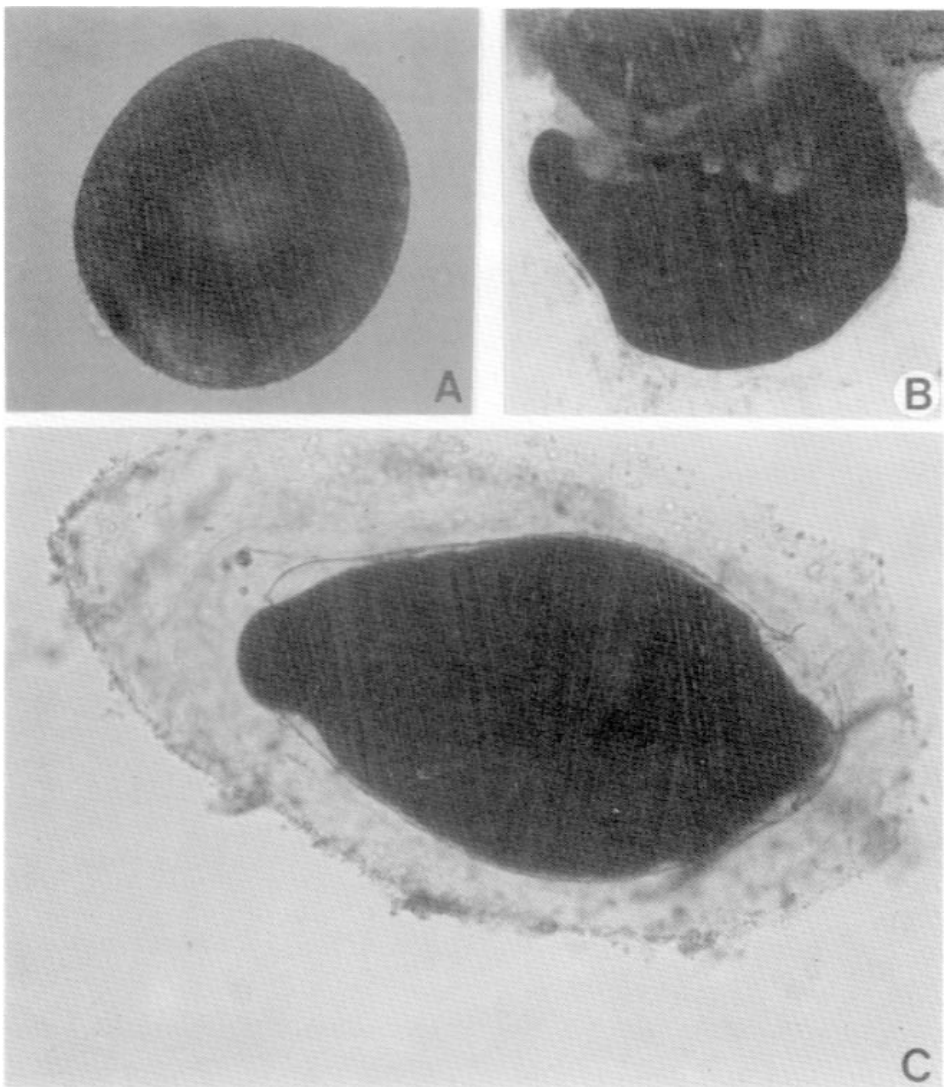

Fig. 3. Photomicrographs of parasitic ciliates in diseased flounder.
A & B. Fresh wet preparation of ciliate(×100).
C. A parasitic ciliate within a skin mucus(×100).

In the experimental infections under laboratory conditions(16℃: 32±2‰), 3 fish of ten juvenile showed white spots on the body surface 4 days after exposure. Free-swiming ciliates released from infected juvenile were collected by pipetting and observed with a light microscope. The results revealed that the ciliates have identical reproductive characteristics as mentioned above.

Discussion

Table 2. Effects of temperatures on ciliate survival at various salinities

Reproductive Pattern	No. of collected	Temp. (℃)	Salinity(‰)							
			0[3]	5	10	15	20	25	30	35[4]
Atypical[1]										
	54	12	−	−	−	−	−	⊕	⊕	⊕
	60	16	−	−	−	−	⊕	⊕	⊕	⊕
	52	20	−	−	−	−	⊕	⊕	⊕	⊕
	50	24	−	−	−	−	−	−	⊕	⊕
Typical[2]										
	106	12	−	−	−	−	−	−	−	⊕
	100	16	−	−	−	−	−	⊕	⊕	⊕
	108	20	−	−	−	−	⊕	⊕	⊕	⊕
	110	24	−	−	−	−	−	−	⊕	⊕

[1] and [2] Refer to Table 1.
[3] Unchlorinated tap water.
[4] Sea water.
− Division process interrupted:endoplasm degeneration.
⊕ No apparent damage caused to the ciliate.

White spot disease of marine fish, in terms of cryptocaryoniasis was first recorded in Japan in 1937 by Skima. Later, the causal organism of the disease was named *Cryptocaryon iirritans* by Brown(1951). The disease is now thought to occur worldwide(Lom and Dykova, 1992), and also the parasite is recurring problem in marine fish in aquaria and is being a concern in commercial mariculture(Coloni, 1985).

Many workers have thought that the disease was significantly affected by water temperatures. Skima(1937) reported that the disease showed frequent occurrences from May to November at water temperature of 15-25℃. Nigrelli and Ruggieri(1996) and Wikie and Gordin(1969), however, found that the disease would not develop at a temperature below 19℃ in aquaria, but most commonly develop in aquaria kept at 20-25℃. Recently, Diggles and Lester(1996) reported that the haviest infection of *C. irritans* on *Acanthopagrus austrails* occurred at 17℃ and found that the infection was maintained as ever at 15℃.

Our findings that the disease occurred at low temperature(<16℃) during the winter corresponded to the reports of Skima(1938) and Diggles and Lester(1996). This indicates the possible existence of different strains or species of *C. irritans* which can be differentiated by their temperature tolerance, as has been proposed by some authors(Diamant *et al.*, 1991; Diggles and Lester, 1996). According to the report of Nigrelli and Ruggieri(1996), the water temperature at Red Sea seldom drops below 19℃ in winter, but later, other authors reported

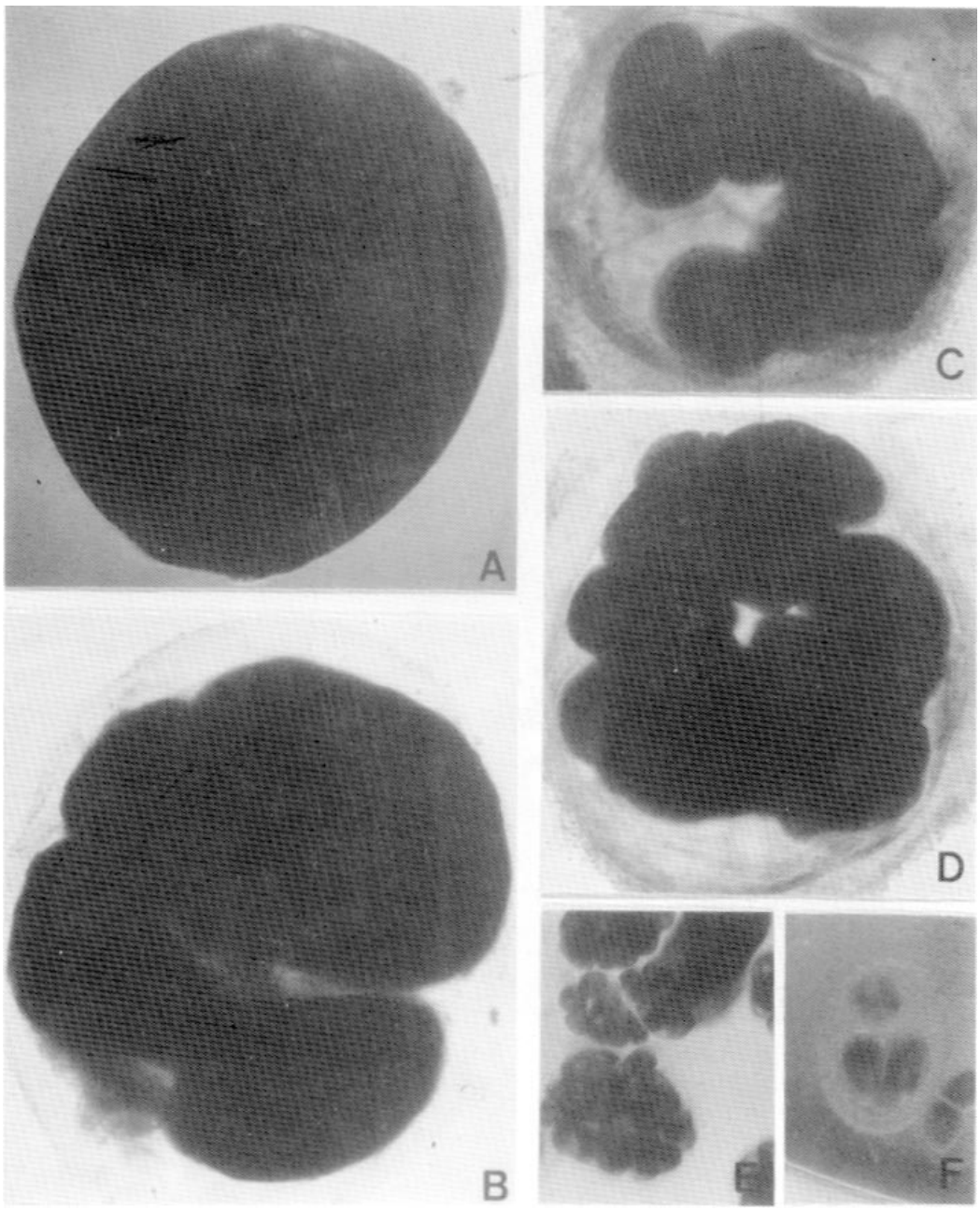

Fig 4. A feature of typical form in parasitic ciliates.
A. A free swiming ciliate. ×200.
B. ciliate showing cyst wall. ×200.
C & D. Ciliate showing palintomic division within thick cyst. ×200.
E. Multi-cell stage. ×100.
F. Ruptured cyst with released tomite. ×100.

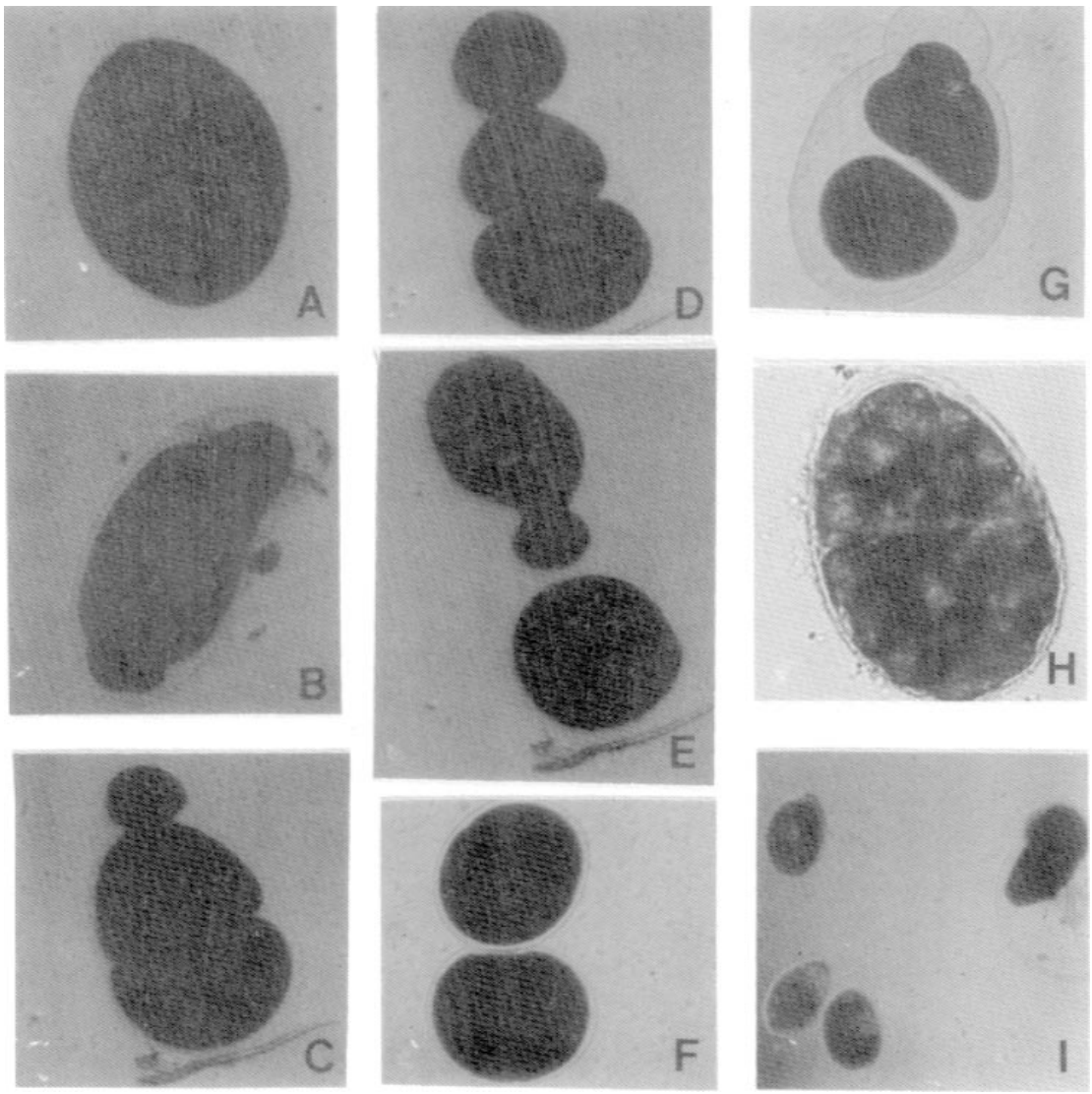

Fig 5. A feature of atypical form in parasitic ciliates.
A. A free swiming ciliate. ×40.
B. Ciliate showing cyst wall. ×40.
C, D and E. Ciliate showing budding within cyst. ×40.
F & G. Ciliates forming cyst wall again. ×100.
H. Ciliate showing multiple fission within cyst. ×200.
I. Ruptured cyst with released tomite. ×100.

the water temperature of the winter ranged from 15 to 28℃. The ciliates isolated in this study have more tolerance to low temperature than other isolates.

There is limited information on the pathologic changes which is associated with white spot disease of marine fish. In gross pathology, Nigrelli and Ruggieri(1996) observed

Table 3. Effects of temperatures on tomite development at constant salinity(35‰)

Reproductive pattern	No. of collected	Temp. (℃)	No. of encysted/ excysted	Excysted time(day)	Percentage of excystment
Atypical[1]					
	30	12	–	14–17	–
	30	16	24/23	13–15	76.6
	29	20	26/21	11–13	72.2
	32	24	13/10	7–13	31.6
Typical[2]					
	60	12	22/18	8	30
	60	16	38/30	5–8	50
	61	20	49/46	4–7	75
	58	24	15/6	6–8	10

[1] and [2] Refer to Table 1.

that the basic response of the host to *C. irritans* infection was excessive mucus production. They also found skin ulcers owing to severe infection. In this study we observed that the moribund flounders showed excessive mucus production and hyperplasia of epithelial cells in the gill as previously discribed. Smith(1929) mentioned that epithelial hyperplasia causes fishes to become oxygen-depleted. Huges(1970) suggested that excessive mucus production of fishes interferes with osmoregulatory function because fish mucus is relatively impermeable to water and ions. As the above indicates, it was believed that the moribund flounder died of laboring breath.

There have been numerous reports on morphology and life cycle of *C. irritans*. According to many authors, infective theronts are pyriform and 25-60㎛ in diameter(Brown, 1963; Nigrelli and Ruggieri, 1966; Cheung *et al.*, 1979; Colorni, 1987; Colorni and Diamant, 1993) and feeding trophonts are spherical and 60-450㎛ in diameter(Cheung *et al.*, 1979; Colorni, 1985; Matthews *et al.*, 1993). The macronucleus of the trophont has four lobes arranged into a crescent(Brown, 1963). In addition, Dikerson and Dawe(1995) remarked that macronucleus of live unstained trophont was highly diffcult to see, because the cytoplasm of *C. irritans* trophont was very opaque. The reproduction of *C. irritans* occurred as free swiming tomonts as previous studies(Brown, 1963; Nigrelli and ruggieri, 1966; Cheung *et al.*, 1979; Colorni, 1987; Colorni and Diamant, 1993). In brief, the mature trophont leaves the fish and it forms a cyst. The encysted parasite(tomont) undergoes a series of palintomic divisions producing daughter cells(tomites) which differentiate into infective parasites(theronts).

In contrast to the results of previous studies, we found that the ciliates isolated from the

flounder in low temperatures(<16℃) were distinguished into two forms. The one form showed the same characteristic as *C. irritans*, while the other form was somewhat larger in size than that of *C. irritans* and had only one macronucleus in live state. And also, the latter form reproduced by not only palintomic division but also budding. It was difficult to distinguish these ciliates by morphological features, but they were obviously distinguished by the reproduction pattern. It was thought that these ciliates isolated from the flounder in low temperatures were a strain of *Cryptocaryon*, same as the previously reported *Cryptocaryon* - like ciliate from cool waters by Diament *et al.* (1991). More criticial works are needed on the morphology and reproductive biology of *Cryptocaryon* sp. depending on the host origin.

It was thought that the water temperature and salinity influenced greatly on the growth and devlopment of *C. irritans*. The trophont remains on the fish for 3 to 7 days and the tomont reproduction required a period outside of the fish for 3 to 28 days at 24-27℃(Colorni, 1985; Burgess and Matthews, 1994; Yoshinaga and Dickerson, 1994). Colorni(1985) reported that all tomonts degenerated when immersed in 0 to 15‰ for 48 h. Skima(1961) and Nigrelli and Ruggieri (1996) reported that the optimal temperature of this parasite was 20-23℃ and 22-25℃, respectively, and incubation period of tomont was 6-9 days in optimal temperature. On the other hand, Cheung *et al.*(1979) found that the opitimal temperature for exycystment was 30℃ and 100% of tomont excysted in 7 days at the same temperatures, and no excystment occurred at 37℃ and 7℃. They also found that low salinity(<16‰) caused cytolysis of tomont. According to Diggles and Lester(1996), at 20℃ trophonts stayed on the fish longer, tomonts took longer to excyst, and the resulting theronts were larger than at 25℃. They thought that *Cryptocaryon* exhibited variability in morphometric on different hosts and under different temperature conditions.

We found that the one form of ciliate showed the same characteristics of *C. irritans* corresponding to previous results, but the other form of ciliate was not so. The other form of ciliate(atypical) had better viability at low salinity and longer excystment time than that of the one form of ciliate(typical). This results indicated that the other form of ciliate(atypical) had a high resistance against environmental conditions such as low temperature or salinity.

It is clear that white spot disease of embankment-farmed flounder occurs not only in higher temperature than 20℃ but also in lower than 16℃. The parasites, however, show two forms of life cycle at low temperatures(<16 ℃). It is not quite clear why they show additional form at lower temperature and whether the different forms are in fact different strains or not in present study, requring furher study.

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저수온 양식 넙치 *Paralichthys olivaceus*의 Cryptocaryoniasis

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1995년 겨울 경북 구룡포에 있는 축제식 양식장에서 사육중이던 넙치(평균 체장 : 31㎝, 평균체중 : 300g)에 백점병이 발생하여 대량 폐사를 일으켰다. 감염 넙치로부터 병리조직학적, 원인충의 형태학적 및 생물학적 특성을 조사한 결과, 아가미 및 지느러미의 병리조직학적 검사에서 많은 섬모충들이 상피 하층으로 뚫고 들어감으로써 기생 부위의 상피 세포와 점액 세포가 증식을 일으킨 것을 볼 수 있었고 병어의 지느러미, 체표 및 아가미에서 작은 백점을 나타낸 충체는 기생성 섬모충 *Cryptocaryon irritans*의 특징을 갖추고 있었다. 그러나 이 충의 증식 방법은 다분열증식법 이외에도 출아와 다분열을 혼합한 또하나의 증식법(혼합 증식법)을 가진 것으로 조사되었으며 수온 16℃에서는 위와 같은 두가지의 증식 방법이 약 3대 2의 비율로 나타났고 다분열 증식형의 충체는 8~14일 만에, 혼합 증식형의 충체는 13~15일 만에 각각 자충을 방출하였다.

수온 12~24℃의 조건에서 염분 농도 범위 0~35‰를 5‰ 간격으로 조절하여 충체의 생존능력을 살펴본 결과 다분열 증식형의 충체는 12℃에서 30‰이하, 16℃에서 20‰ 이하, 20℃에서 15‰이하 그리고 24℃에서 25‰ 이하에서 각각 폐사된 반면 혼합 증식형의 충체는 12℃에서 20‰ 이하, 16~20℃에서 15‰ 이하 그리고 24℃에서 25‰ 이하에서 각각 폐사를 나타내어 저수온(12~16℃)에서 혼합 증식형의 충체가 다분열 증식형의 충체보다 다소 낮은 염분 농도에서 생존이 가능함을 보여주었다.

수온 12~24℃의 조건에서 탈낭 소요 시간 및 탈낭률은 다분열 증식형의 충체가 12℃에서 8일 및 30%, 16℃에서 6.5일 및 50%, 20℃에서 5.5일 및 75% 그리고 24℃에서 7일 및 10%를 나타낸 반면 혼합 증식형의 충체는 12℃에서 15.5일, 16℃에서 14일 및 76.6%, 20℃에서 12일 및 72.2% 그리고 24℃에서 10일 및 31.6%를 나타내어 모든 수온 조건에서 혼합 증식형의 충체가 다분열 증식형의 충체보다 탈낭 소요 시간이 길며 수온 16℃에서 최고의 탈낭율을 나타내어 저수온에 다소 안정적인 것을 보여주었다.

Key words : Flounder, *Paralichthys olivaceus*, *Cryptocaryon irritans*, Typical form, Atypical form